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(54) Title: METHOD FOR INHIBITING THE INFECTIVITY OF HUMAN IMMUNODEFICIENCY VIRUS (57) Abstract Disclosed herein are methods for inhibiting cell fusion between human T-lymphocytes infected by human immunodeficiency virus (HIV) or free human immunodeficiency virus and uninfected human T-lymphocytes comprising administering inhibitory effective amounts of HIV protein gp120 and CD4-Immuno adhesion and immunogenic compositions for use in the methods.		

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METHOD FOR INHIBITING THE INFECTIVITY
OF HUMAN IMMUNODEFICIENCY VIRUS

Field of the Invention

15 This application is a continuation-in-part application of co-pending U.S. Patent Application Serial No. 287,270 filed December 20, 1988.

The government has rights to this invention by virtue of funding from U.S. Public Service Grant No. RO1-AI28194.

20 One aspect of this invention relates to methods and compositions for influencing the immunogenicity of human immunodeficiency virus (HIV) antigens and more specifically to methods and compositions for raising antibodies that inhibit the propagation of HIV infection.

25 Another aspect of this invention relates to antibodies that have the ability to inhibit such propagation.

Background of the Invention

30 Acquired Immunodeficiency Syndrome (AIDS) is believed to be caused by a retrovirus called human immunodeficiency virus (HIV), also known as HTLV III or LAV. This syndrome is considered responsible for a variety of immunologic abnormalities including but not limited to the depletion and/or selective infection by this virus of helper/inducer (T4) lymphocytes, which results in impairment of the helper/inducer
35 T cell function in affected individuals and in eventual inhibition of normal immune response in such individuals.

The viral envelope (HIV env) includes a population of glycoproteins (called gp 160) anchored in the viral cell

membrane bilayer via their C-terminal region. Each glycoprotein contains two segments: the N-terminal segment, called gp120, which protrudes from the membrane into the surrounding medium; and the C-terminal segment, gp 41, which spans the membrane.

It has been reported that HIV infects CD4⁺ T lymphocytes by a sequence of events beginning with attachment of gp120 to its cellular receptor CD4, a nonpolymorphic surface glycoprotein described in Maddon, P.G. et al, Cell, 42:93-104, 1985; Clark, S., et al, P.N.A.S. (USA), 84:1649, 1987 and Germain, R.N., Cell, 54:441-444, 1988. It is believed that the binding of gp120 to CD4 then triggers anchorage of gp 41 to the lymphocyte membrane, an event in turn followed by cell fusion between the virion and the target lymphocyte; Kowalski, M. et al, Science, 237:1351-1355, 1987; Gallaher, W.R., Cell, 50:327-328, 1987 and Gonzalez-Scarano, F. et al, AIDS Research & Human Retroviruses, 3(3):245-252, 1987.

In addition, HIV infection is propagated by direct lymphocyte-lymphocyte fusion between virus-infected cells (which have been shown to express gp120 and gp 41 on their surface) and uninfected CD4⁺ cells. This fusion takes place even in the absence of free HIV in the surrounding medium. Lifson, J.D. et al, Science, 232:1123-1127, 1986; Sodroski, J. et al, Nature, 322:470-474, 1986; and Lifson, J.D. et al, Nature, 323:725-728, 1986.

The properties and isolation of gp120 from HIV particles and its sequencing from different HIV isolates are well-known and have been extensively described e.g. in the foregoing references. The preparation of gp120 via recombinant DNA techniques has been described in Lasky, L.A. et al, Science, 233:209, 1986 and in published European patent application of Genentech, Inc. published on August 24, 1988, Serial No. 279,688 (based on U.S.S.N. 155,336 filed 02/12/88) naming Berman, P.W.; Gregory, T.J.; Lasky, L.A.; Nakamura, G.R; et al. as inventors, as well as in Landau, N.R., et al., Nature, 334:159-162, 1988; and Lasky, L.A., et al., Cell, 30:975-985, 1987. Finally, isolation of gp120 (whether native or recom-

binant) has been described in Essex, U.S. Patent No. 4,725,669 (2/16/88).

CD4, the cellular receptor of gp120, has been isolated from lymphocytes. Synthetic (soluble) and recombinant CD4 have been described in Smith, D.H. et al, Science, 238:1704-1707, 1987. Other methods as well as properties of CD4 have been described in Jameson, B.A. et al, Science, 240:1335-1339, 1988; Fisher, R.A. et al, Nature, 331:76-78, 1988; Hussey, R.E. et al, Nature, 331:78-81, 1988; Deen, K.C. et al, Nature, 331:82-84, 1988; Trauneker, A. et al., Nature, 331:84-86, 1988; and Lifson, J.D. et al, Science, 241:712-716, 1988.

Two regions of HIV env (gp 160) exhibit the highest immunogenicity that has been observed against this protein:

The first region has been placed between residues 307 and 330 of gp120 and represents an immunodominant epitope since animals immunized with whole gp 160 or gp120 (or with fragments of gp120 containing the epitope) produce high titers of HIV-neutralizing antibodies (i.e., antibodies that inhibit virion-lymphocyte fusion). This immunodominant epitope is situated in a highly variable segment of gp120 that varies from isolate to isolate and, as a result, the antibodies are also isolate-specific. This limits their utility in immunological studies and in therapy against (or prevention of) HIV infection. Also, sera from HIV-infected humans do not contain high titers of these antibodies.

Instead, sera from infected humans contain HIV-neutralizing antibodies (specifically antibodies that inhibit the binding of viral gp120 to lymphocytic CD4) directed to other epitopes of gp120 which have not been precisely identified (though it is thought to be proximal to the CD4-binding site of gp120); Lasky, L.A. et al, Cell, 50:975-985, 1987. The antibodies are group- and not isolate-specific, which indicates that this second epitope is located in a more conserved domain of gp120. However, animals immunized with gp120, gp 160 or various fragments of gp120 have not produced HIV-neutralizing antibodies. Thus, these other epitopes of gp120 appears to be less immunogenic.

Accordingly, one object of this invention is to provide significant amounts of antibodies that neutralize the infectivity of HIV virus (i.e., inhibit its ability to invade T4 lymphocytes).

5 Another object of this invention is to provide antibodies that prevent HIV-induced cell fusion between healthy T4 lymphocytes and lymphocytes infected with HIV.

Yet another object of this invention is to use such antibodies to improve the understanding of the pathogenesis of HIV and to inhibit propagation of HIV infection in human T4 lymphocytes.

Still another object is to provide compositions and methods for raising such antibodies.

15 Further objects of this invention include use of such antibodies in research to elucidate the structure and function of HIV components and the mechanism of HIV infectivity and use of such antibodies in the passive or active immunization of humans for prophylactic or therapeutic purposes.

20 These and other objects of the invention will be apparent to persons skilled in the art in light of the present specification, accompanying claims and appended drawings in which:

Brief Description of the Drawings

25 Figure 1 A-C is a series of plots comparing the magnitude and cell fusion-blocking ability of various antibody titers in successive bleedings of animals immunized with CD4, gp120 and a combination of CD4 and gp120.

30 Figure 2 is a series of graphs illustrating the time course of an experiment with three groups of mice respectively injected with CD4 (A,D,G), CD4-gp120 complex (B,E,H) and gp120 (G,H,I). The weekly serum samples were assayed individually for gp120-binding antibodies (A,B,C), CD4-binding antibodies (D,E,F), and syncytia-blocking capacity (G,H,I). The mice
35 immunized with the complex showed a somewhat higher anti-gp120 response than those immunized with gp120 alone (panels B versus C); a markedly lower titer of CD4 binding as compared to those

receiving CD4 alone (panel E versus D); and a significantly higher syncytia-blocking response (panel H versus G).

Figure 3 is a graph showing titration of 11-week serum from 4 mice injected with CD4-gp120 complex for III_B and RF syncytia-blocking capacity. The parallel behavior of individual sera in the two tests suggests that the antibodies are directed at group-specific determinants.

Figure 4 is a graph showing the effect of antibodies on rCD4-phosphatase binding to solid phase rgp120. In panel A the antibody is OKT4A; in panel B, 94; in panel C, OKT4, in panel D, 55. CD4-phosphatase concentrations: closed circles = 10 micrograms/ml; open circles = 3 micrograms/ml. Ordinate: OD_{405/60} min. Abscissa: antibody concentration, from left to right, 0, 0.3, 1, 3, 10 micrograms/ml.

Figure 5 is a graph showing effect of rgp120 on rCD4-phosphatase binding to various antibodies captured on solid phase goat anti-mouse Ig. In panel A the antibody is OKT4A; in panel B, 94; in panel C, OKT4; in panel D, 55. Concentration of rCD4-phosphatase: closed circles = 10 micrograms/ml; open circles = 1 microgram/ml; squares = 0.1 microgram/ml. Ordinate: OD_{405/60} min (A,B) or OD_{405/120} min (C,D). Abscissa: concentration of gp120 (0, 0.4, 4, 40 micrograms/ml).

Figure 6 is a graph showing the effect of rgp120 on phosphatase-labeled antibody binding to solid phase rCD4. In panel A, the antibody is 94; in panel B, 55. Open circles = no rgp120; closed circles = rgp120 at 0.1 microgram/ml; squares = rgp120 at 1.0 microgram/ml. Ordinate: OD_{405/60} min. Abscissa: antibody concentrations (0.1, 1, 3, 10 micrograms/ml).

Summary of the Invention

It has now been discovered that antibodies raised pursuant to immunization with a complex of (a) the HIV antigen gp120 and (b) CD4-Immunoaderisin have the ability to inhibit propagation of HIV infection to healthy human T-lymphocytes (by inhibiting lymphocyte-lymphocyte fusion and invasion of lymphocytes by HIV).

Detailed Description of the Invention

As used in this disclosure, each of the following terms shall have the meaning ascribed to it below.

5 "gp120" shall mean not only gp120 itself but also any other molecule that binds with CD4 in a similar manner and that when so bound has the same conformation. For example, the term will include fragments of gp120 that bind to CD4 as well as analogs and derivatives of gp120 that possess the ability to bind CD4 and to generate antibodies with the cell-fusion
10 blocking ability of the antibodies of the present invention. In addition, this definition of gp120 shall include gp120 from any HIV isolate since methods for sequencing this protein are known and are independent of the particular isolate of HIV from which the native protein is derived.

15 "CD4" shall mean CD4 and/or fragments, derivatives or analogs containing the gp120-binding site of CD4, such as CD4 isolated from the lymphocytic surface and CD4 or CD4 derivatives (such as CD4-Immunoaderhin and analogs (e.g. soluble CD4) produced by synthetic (including but not limited to recombinant
20 DNA) techniques.

"gp120/CD4 complex" shall mean a bimolecular (i.e., noncovalent) conjugate or complex between gp120 and CD4 (or between gp120 and antiidiotypic antibody bearing a CD4 internal image). A simple mixture of gp120 and CD4 contains this
25 complex because of the high affinity between gp120 and its cellular receptor. It is not necessary that the complex be made of isolated gp120 and CD4. For example, whole lymphocytes having gp120 bound to the CD4 on their surface are envisioned as a possible form of an immunogen encompassing the gp120/CD4
30 complex of the present invention. The native lymphocytes of an HIV-infected human would not act as such an immunogen in that human because of the ability of autologous CD4⁺ lymphocytes to act as antigen-presenting cells (APC), as reported by Lanzavecchia, A. et al., Nature, 334:530-532, 1988. (Consequently, the
35 gp120/CD4 complex even if it exists on the surface of these human lymphocytes could not act as an immunogen.) However, antibodies of the type of the present invention could be

induced in a human by immunization with the foregoing complex in one or more of its forms contemplated herein.

5 "Antibodies of the present invention" or "the present antibodies" shall mean (a) polyclonal, group-specific antibodies raised by immunization with the gp120/CD4 complex and capable of inhibiting lymphocyte fusion; and/or (b) monoclonal antibodies raised against this immunogen and possessing the same fusion inhibiting property.

10 "Neutralization of HIV" shall mean inhibition of the ability of HIV to bind to and invade a susceptible lymphocyte.

"Cell fusion inhibition" shall mean inhibition of the ability of HIV-infected lymphocytes to form syncytia with healthy CD4⁺ lymphocytes (i.e. lymphocytes possessing surface CD4) in the absence of free HIV.

15 As stated above, although HIV-infected humans do produce antibodies to gp120 that inhibit the binding of HIV to CD4 and hence prevent invasion of CD4⁺ T lymphocytes (i.e., lymphocytes possessing surface CD4) by the virus, the titers of such antibodies are not very high either in absolute terms or
20 by comparison to these individuals' titers of nonneutralizing antibodies.

The majority of sera from seropositive individuals contain some HIV-neutralizing antibodies directed against an epitope located within a more conserved area of gp120, and
25 distinct from the immunodominant epitope. Such antibodies are group-specific and constitute useful investigative tools in the pathogenesis of HIV and in research efforts to produce abatement or prevention of HIV infection. However, even though these human group-specific antibodies neutralized HIV in vitro
30 and inhibited lymphocyte fusion in vitro, they failed to inhibit lymphocyte invasion by free HIV introduced in the serum of primates passively immunized with the human antibodies. This failure has been attributed to the fact that these antibodies are not directed towards an immunodominant epitope
35 and/or to the insufficient affinity of these antibodies for their antigenic determinant compared with the extremely high affinity of gp120 for its lymphocytic receptor CD4 (the

equilibrium constant of the latter is of the order of 10^{-9} M which is significantly higher than the affinity of most antigens for their antibodies). Also, nonprimate mammalian immune systems do not recognize this epitope of gp120 and consequently animals do not produce such antibodies. Therefore, the need still exists for different antibodies that (a) are group-specific, (b) neutralize the virus and inhibit cell fusion and (c) do not compete with the high-affinity binding of gp120 to CD4.

In accordance with the present invention, comparative experiments were conducted in which animals were immunized with (a) CD4, (b) gp120, and (c) a mixture of CD4 and gp120 which is believed to result in a bimolecular (noncovalent) conjugate or complex between CD4 and gp120 because of the high affinity (10^{-9} M) of gp120 for its receptor.

Animals immunized with CD4 alone exhibited a very high titer of anti-CD4 antibodies. However, only a relatively very small portion of these antibodies inhibited the ability of HIV infection to spread among T4 lymphocytes as measured by a cell fusion assay which tests the ability of HIV-infected lymphocytes to fuse with healthy lymphocytes bearing CD4.

In parallel, another group of animals were immunized with gp120 alone. The anti-gp120 immune response was significant (although not nearly as high as the anti-CD4 response) but the anti-gp120 had no HIV-neutralizing ability (as measured by the same assay).

A third group of animals were immunized with a mixture of CD4 and gp120. The immune response showed the presence of anti-CD4 antibodies (although the titer was significantly lower compared to the anti-CD4 elicited by immunization with CD4 alone) and anti-gp120 antibodies (in amounts comparable to those elicited by immunization with gp120 alone). However, the sera showed a very high titer of cell-fusion inhibiting antibodies.

The antibodies of the present invention are not simply anti (CD4), since the cell-fusion inhibiting titer does not correlate with the titer obtained by the immunization with CD4

alone and since anti CD4 do not possess the cell-fusion inhibiting ability of the antibodies of the present invention. For the same reasons, the antibodies of the present invention do not appear to be simply anti gp120. Furthermore, the present antibodies are not the same as previously observed antibodies which inhibit the event of binding between CD4 and gp120 because the titers of the present antibodies do not correlate with the titers of the previously observed binding-inhibiting antibodies. Finally, the antibodies of the present invention are not elicited except in the presence of the gp120/CD4 complex, as will be illustrated below, and therefore constitute novel and distinct entities.

The HIV-neutralizing ability of the antibodies of the present invention has been measured by a cell-fusion assay developed by Skinner, M.A. et al, J. Virol., 62:4195, 1988. This assay exploits the ability of HIV-infected lymphocytes to form syncytia (fused cells) with healthy but HIV-susceptible (CD4⁺) lymphocytes (in the absence of free HIV), a process that starts by the expression (on the surface of infected lymphocytes) of gp120, which then binds to the CD4 of healthy cells. The assay thus compares the ability of HIV-infected lymphocytes to form such syncytia under experimental conditions with the ability of the infected cells to form syncytia under control conditions, i.e. in the absence of a potential fusion-inhibitor.

This assay is a stringent indicator of HIV-infection inhibition ability by a given inhibitor and in particular by an antibody. Another fusion assay is available that measures the ability of free HIV to invade lymphocytes (i.e., the fusion takes place between the virion and the lymphocytes). However, although many antibodies are available that can inhibit lymphocyte infection by free virus, very few antibodies also inhibit lymphocyte-lymphocyte fusion in the absence of free virus. On the other hand, most, if not all, antibodies that inhibit cell fusion also inhibit infection by free virus.

For these reasons, the performance of the present antibodies in the lymphocyte-lymphocyte cell fusion assay

constitutes good evidence of the HIV-inhibiting ability of such lymphocytes.

5 The antibodies of the present invention may be thus used to inhibit both invasion of lymphocytes by HIV and spread of HIV infection via lymphocyte fusion and hence constitute good candidates for passive immunization (both prophylactic and therapeutic). Such passive immunization may be combined with cytotoxic agents or coadministered with other HIV inhibitors, such as ricin toxin A chain which when linked to recombinant CD4 has been shown to be selectively toxic to infected T-lymphocytes. Till, M.A., et al, Science, 242:1166-1168, 1988.

10 In addition, immunization of susceptible mammals with a combination of gp120/CD4 is expected to improve the effectiveness of the immune response of these mammals against HIV infection both preventively and therapeutically.

15 Other uses for the antibodies of the present invention include use in screening tests for the presence of the gp120/CD4 complex; as research tools to identify new epitopes of gp120 and specifically epitopes that are available only by changes in the conformation of gp120 by CD4 binding and/or vice versa. Monoclonal antibodies in accordance with the invention and especially human monoclonal antibodies represent a preferred form of the present invention and can be used for passive immunization in humans.

20 In addition, many other uses are contemplated as will be apparent to those of ordinary skill in the art.

25 Amounts used for immunization in mammals can generally vary from about 10 to about 100 micrograms CD4/kg body weight and from about 13 to about 130 micrograms of gp120/kg body weight. The foregoing amounts are based on the assumption that equivalent amounts of CD4 and gp120 will be used, which is preferred but not necessary. It will be appreciated of course by those of ordinary skill in the field that an excess of one or the other constituent of the complex (i.e., an amount in addition to that sufficient to form a complex with the available amount of the other constituent) is not fatal to the operability of the present invention but an equimolar mixture

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of CD4 and gp120 is preferred.

Well-known immunization protocols may be used with or without adjuvant. One preferred protocol involved use of the complex in complete Freund's adjuvant as set forth in Example 1 (of course any other well-known immunization adjuvant can be used or adjuvant can be omitted altogether).

A single immunization is sufficient, but immunization may be repeated 4 weeks after the first injection with an additional optional booster 4 weeks after the second injection in incomplete adjuvant (or without adjuvant). In addition, the immunogenic ability of the complex of the present invention can be boosted by use of carriers such as tetanus toxoid, keyhole limpet hemocyanin, vaccinia virus, diphtheria toxoid, etc., as is well known in the art.

Concentrations of the antibodies of the present invention effective in inhibiting lymphocyte-lymphocyte fusion should be at least sufficient to prevent successful carrying out of the sequence of events that lead to either invasion of the lymphocytes by free HIV or lymphocyte-lymphocyte fusion between any available gp120 (whether on the viral envelope or on the surface of an infected lymphocyte) and CD4⁺ lymphocytes. The upper limit of the effective concentration is irrelevant in vitro. In vivo, the upper limit of the effective antibody concentration may be limited by factors outside the binding mechanism, such as on immune response of the host against the antibodies.

The antibodies of the present invention (whether monoclonal or polyclonal) may be purified by well-known techniques for purification of immunoglobulins, including but not limited to use of precipitation techniques (such as ammonium sulfate precipitation) and/or immunoaffinity chromatography methods (with an antigen as the adsorbent) wherein the desired antibody is preferentially bound to the column or excluded in the eluant; protein A sepharose chromatography; Affigel-blue chromatography; high performance liquid chromatography and combinations of these techniques.

Monoclonal antibodies to the complex of the present

invention may be raised according to well-known techniques, such as those described in Kohler and Milstein, Nature, 256:495, 1975 and in Goding, infra.

In addition, it is believed that human monoclonal antibodies may be raised from immortalized human lymphocytes sensitized against the complex of the present invention in vitro (as described in Reading, C.L., 1982, J. Immunol. Meth., 53:261; Hoffman, M.K. et al in Engleman, E.G. et al (Eds) Human Hybridomas and Monoclonal Antibodies, Plenum Press, New York, 1985, p. 466; Borrebaeck, C.A.K., Trends in Biotechnology, 4:147, 1986) or in vivo (by immunizing humans with the complex as described herein and selecting B-cells with the appropriate specificity). Human C3D⁺ peripheral blood mononuclear lymphocytes can be exposed to Epstein-Barr virus for immortalization. Epstein-Barr virus (EBV) can be obtained from the filtered supernatant of the marmoset cell line B95-8 (Miller, G., and Lipman, M., 1973, PNAS (USA) 70:190) available from the ATCC under Accession No. CRL-1612. Infected lymphocytes are then washed and cultured in RPMI-1640 medium in the presence of fetal bovine serum, glutamine, penicillin and streptomycin in 96-well plates at 10⁴ cells per well. After screening for antibody production, positive cultures can be expanded and cultured further. Cultures with supernatants showing specific reactivity to the complex of the present invention can be subcultured on feeder layers of GK5 human lymphoblastoid cells (derived from GM1500 as described in Kearny, J., N. Engl. J. Med., 309:217, 1983) irradiated with 3000 rads of gamma-radiation. Stable clones can then be subcultured at low densities (10-100 cells) on feeder cells and the subcultures can be expanded. The specificity of the antibodies can be tested by the Skinner et al assay referenced and described herein.

Antiidiotype antibodies can be obtained by immunizing syngenic mice with anti-CD4 monoclonal antibody. A most efficient protocol of immunization is to inject the monoclonal antibodies four times a day at weekly intervals. The total amount injected is 13 doses of 20 micrograms per mouse at each

time. In particular, on day 1, the antibody is injected coupled to KLH (keyhole limpet hemocyanin) in complete Freund's adjuvant; on day 6 the antibody is injected coupled to KLH in incomplete Freund's adjuvant (IFA); on day 13 the antibody is injected coupled to KLH in saline; on day 27 the antibody is injected alone in incomplete Freund's adjuvant. To obtain the monoclonal antibodies, the animals are boosted after 4 to 12 weeks after the last injection by injecting again the monoclonal antibody in incomplete Freund's adjuvant in saline. Spleen fusion with an appropriate myeloma partner is performed 3 days after the boost. Fusion and hybrid growth and selection are then performed in accordance with well-known techniques. The antiidiotypic antibodies bearing CD4 internal image can be identified by testing for binding to gp120 (e.g., by radioimmuno assay, enzyme-linked immunosorbent assay, etc.) and purified using well-known methods including immunoaffinity chromatography using gp120 as the adsorbent.

Again, the complex of the present invention when antiidiotype antibodies ("AA") are used can be formed by mixing gp120 and AA preferably in equimolar amounts and waiting for the two constituents to complex with each other.

The materials used in the present invention may be purified from natural sources or synthesized by well-known (recombinant and other) techniques as described above. In addition, recombinant CD4 and CD4-Immunoaderisin are available from Genentech, Inc. and so is recombinant gp120. If not already obtained in purified form, these materials should preferably be purified prior to use in immunization. Techniques for purification are well-known; see, e.g., U.S. Patent No. 4,725,669 of Essex et al. issued February 16, 1988.

To be effective in inhibiting cell invasion by HIV and cell fusion, the antibodies of the present invention must be present in the vicinity of infected T4 lymphocytes (or HIV-susceptible uninfected T4 lymphocytes) before the gp120 expressed on the surface of infected T4 lymphocytes (or the gp120 on the surface of the virion) binds to its receptor (CD4) on the surface of an uninfected lymphocyte (or on the surface of a

susceptible lymphocyte in the case of infection by free virus). Preferably, the antibodies of the present invention will be present in such vicinity prior to encounter between the gp120-bearing infected lymphocyte (or virus) and the target CD4⁺ lymphocyte.

The invention is further described in the Examples that follow. The purpose of these examples is to illustrate the present invention and not to limit its scope.

10 Example 1: Immunization of Mice and Antibody Titers

Three groups of 4 mice (each weighing 25 g) were immunized as follows:

15 The first group was injected once intraperitoneally (i.p.) with 10 micrograms of CD4 in 0.2 ml of complete Freund's adjuvant.

The second group was injected once i.p. with 12.5 micrograms of gp120 in 0.2 ml of complete Freund's adjuvant.

20 The third group was injected once i.p. with a mixture of 10 micrograms of CD4 and 12.5 micrograms of gp120 (previously incubated together for 30 minutes) in 0.4 ml of complete Freund's adjuvant.

25 The mice were bled weekly over a three-month period and the sera were monitored for CD4- and gp120-binding titers. In addition, the sera were monitored for their ability to block lymphocyte-lymphocyte fusion, according to an assay described in Example 2 below, and for their ability to inhibit binding between CD4 and gp120, according to an assay described in Example 3.

30 CD4 and gp120 titers were determined as follows:
Binding antibodies were assayed by ELISA. Microtiter plates were coated with the appropriate antigen (CD4 or gp120, respectively) at a concentration of 3 micrograms/ml in carbonate buffer 0.1 M, pH 9.6 overnight at 4°C, washed and blocked by incubating them with 1% bovine serum albumin (BSA)
35 in phosphate buffer saline (PBS) for 45 min. at room temperature. A 0.1 ml sample of test serum at various dilutions in PBS were then added and incubated for two hours at room

temperature. The plates were then treated with goat-antimouse antibodies labelled with alkaline phosphatase and finally incubated with PNPP (disodium p-nitrophenyl phosphate) at a concentration of 1 mg/ml in diethanolamine buffer, pH 9.8. Each step was followed by a wash in PBS-Tween 20 buffer. Absorption at OD₄₀₅ was determined 1 hour after incubation with substrate in a Titertek Multiskan MCC 340. The OD readings were converted to micrograms/ml of undiluted serum by interpolation to OD=0.5 and assuming a 1:1 ratio between bound mouse antibody and goat and mouse tracer.

Figure 1A is a plot of the anti-gp120 titer (in micrograms/ml serum) of sera elicited by immunization of mice with gp120 (solid line), CD4 (broken line) and the gp120/CD4 complex (---) for each weekly bleeding.

As evident from Figure 1A, immunization with gp120 alone elicited anti-gp120 antibodies; immunization with CD4 alone elicited no anti-gp120 antibodies; immunization with the complex also elicited anti-gp120 antibodies (i.e. the complex generated formation of antibodies against gp120 alone).

Figure 1B is a plot of the anti-CD4 titer (in micrograms/ml serum) of sera elicited by immunization with CD4 alone (broken line), gp120 alone (solid line) and complex (---).

In Figure 1B, immunization with CD4 alone elicited a very high anti-CD4 immune response; immunization with the complex elicited a lower but significant anti-CD4 response; and immunization with gp120 alone elicited no anti-CD4 response except after week 7 when a small antiidiotype (anti-(anti gp120)) response was observed.

Example 2: Lymphocyte Fusion Assay

The assay to determine the immune serum capacity to block lymphocyte-lymphocyte fusion was described in Skinner, supra. Ten microliters of different dilutions (at least 1:10) of the test serum were distributed in Costar 96 A/2 (half-well) plates.

5x10³ or 10x10³ infected cell partners (from CB/IIIB or

CEM/RF lymphocyte cell line publicly available from National Institute of Allergy and Infectious Disease, Reagent Program respectively infected with HTLV IIIB or HTLV RF isolate available from Dr. Gallo at the National Institute of Health) were added to each well contained in 40 microliters of culture medium. Then, 7×10^4 uninfected CD4⁺ human lymphocytes (Molt4 available from American Type Culture Collection, Rockville, MD, Accession No. CRL-1582) in 40 microliters of culture medium containing fetal bovine serum were added to the wells and the plates were incubated at 37°C in a 5% CO₂ atmosphere for 20-24 hours. The plates were then read for the occurrence and the number of lymphocyte syncytia in an inverted microscope at a 40-fold enlargement. Giant cells having a size of at least five times the area of normal cells were scored as syncytia produced by cell fusion. In the absence of any antiserum, the syncytia were 50-80/well (control). Fusion-blocking units (FBU) were calculated by converting the percent decrease in syncytia scored relative to the control value and taking into account the serum dilution (1 FBU is defined as the amount of antibody that reduces the number of syncytia to 50% of the control value). The results, in FBU, are plotted in Figure 1C. Again, the solid line represents FBU achieved by immune sera of mice immunized with gp120 alone. In Figure 1C, this value is essentially the same as the control. The broken line represents the fusion-blocking ability of immune sera elicited by immunization with CD4 alone. In Figure 1C, this value is positive but not very high. This is attributable to the fact that anti-CD4 will bind some of the CD4 on the uninfected lymphocyte surfaces and thus prevent the gp120 of the infected lymphocytes from binding to the CD4.

In Figure 1C, the line - - - - - represents the fusion-blocking ability of immune sera elicited by immunization with gp120/CD4 complex. The FBU of this sera starts at about that of the anti-CD4 sera in week 1 and extends to about 50 times that of the anti-CD4 sera.

Example 3: Monoclonal Antibodies

Monoclonal antibodies will be raised by immunizing mice with complex in accordance with the method of Example 1 (except that two injections can be used spaced 4 weeks apart) optionally with a booster using incomplete Freund's adjuvant 4 weeks after the second injection. Three days after the last immunization, spleen cells will be obtained, purified and fused with myeloma cells. The spleen of the mouse with the highest fusion-blocking ability will be excised using well-known dissociation techniques. A single-cell suspension will be made up by teasing the spleen as described in Goding, J.W., Monoclonal Antibodies: Principles and Practice, Academic Press, Inc., New York 1983, pp. 50-97 and specifically on p. 64. The spleen cells will be harvested by centrifugation (e.g. 400xg for 5 min.) and washed. Erythrocytes will be removed by ammonium chloride lysis followed by centrifugation. The spleen cells will be counted and approximately 10^8 cells will be used for fusion with commercially available mouse myeloma cells (e.g. SP 20 from American Type Culture Collection under Accession No. CRL-1581). $(2 - 3) \times 10^7$ myeloma cells will be mixed with the spleen cells in serum-free media and centrifuged at 400xg for 5 min. Any remaining medium will be removed by suction. The cell pellet will be suspended in 0.5-1 ml of warm fusion medium containing 10 g of 50% w/v PEG (m.W. 1500) and 10 ml Dulbecco's modified Eagles' Minimum Essential Medium, pH 7.6. The mixture will be stirred, centrifuged and resuspended in fetal bovine serum-containing medium using normal spleen cells as feeders. The cells will then be exposed to HAT selective media and grown in such media in an atmosphere containing 5% CO₂. The cultures will be pulled and fed. Hybrids will be growing and screenable at 10-15 days after incubation begins. Positive clones (i.e. clones secreting fusion-blocking antibody) will be identified and recloned until their secretion of the desirable immunoglobulin is steady and reliable. Monoclonals will thus be obtained. The Skinner et al assay can be used to determine specificity of the desired antibody.

Difficulty in obtaining such monoclonals is not expected because the titers of fusion-blocking antibody are relatively high as demonstrated in previous Examples. If desired, spleen cells secreting anti-CD4 and/or anti-gp120 will be separated first before fusion to maximize the probability of obtaining fusion-blocking monoclonals.

Example 4:

It is known that the HIV infection of CD4⁺ cells can be prevented by antibodies specific for the gp120 binding site on CD4, defined as the V1 domains of CD4 involved in the initial CD4-gp120 binding event, i.e., the region homologous to CDR-2, amino and residues 41-52 (Peterson, A., et al., Cell. 54:65, 1988; Landau, N.R. et al., Nature. 334:159, 1988; Clayton, L.K., Nature. 335:363, 1988; Jameson, B.A. et al., Science. 240:1355, 1988), and in part CDR-3, amino and residues 83-92 (Nara, P.L., et al., Proc. Natl. Acad. Sci. USA. 86:7139, 1989; Sattentau, Q.J., et al., J. Exp. Med. 170:1319, 1989). These antibodies prevent infection by sterically interfering with the binding site.

Poly- and monoclonal antibodies from mice immunized with CD4 complexed to gp120, their binding characteristics and capacity to prevent the formation of HIV-dependent syncytia, are described below. The data presented below demonstrate that there are epitopes on CD4, unrelated to the binding site of gp120, which antibodies can recognize, thus affecting post-virus-binding events that usually lead to infection.

The following materials and methods were used as described below.

Recombinant molecules. Soluble recombinant CD4 (rCD4), CD4 immunoadhesin (containing V₁ and V₂ domains of CD4 spliced to the CH2 and CH3 domains of human IgG as described in Byrn, R.A., et al., Nature 344:667, 1990, and recombinant gp120 were obtained from Genentech Inc., South San Francisco, CA.

Animals. Balb-c female mice 10-15 weeks old (Jackson Labs, Bar Harbor, ME) were used both for immunization and for production of monoclonal antibodies.

Immunizations. Mice were injected intraperitoneally (i.p.) with antigen emulsified in Complete Freund's Adjuvant (CFA, Difco, Detroit, MI). The antigens and doses were a) CD4, 16 micrograms/mouse; b) gp120, 12.5 micrograms/mouse, c) CD4-gp120, 16 micrograms CD4 and 12.5 micrograms gp120, thoroughly mixed and incubated for 20 min, and then emulsified in CFA. The mice were bled prior to immunization and every week after, for 13 weeks. Serum samples were stored at -20°C.

Enzymes and substrates. Alkaline phosphatase and glutaraldehyde, used to label antibodies for Enzyme Linked Immunosorbent Assay (ELISA) tests, and substrate paranitrophenylphosphate (PNPP) were acquired from Sigma Chemicals (St. Louis, MO).

Monoclonal antibodies. The monoclonal antibodies (mAbs) mentioned below are F-91-36, F-91-55 and F-91-94, hereinafter called 36, 55 and 94 respectively. They were derived from a fusion of mouse 91, immunized with CD4-gp120 complex as described above. All these mAbs are IgG₁ subclass.

Antibody binding of gp120 and CD4. ELISA tests were performed by coating plates with 10 micrograms/ml gp120 or, respectively, with 3 micrograms/ml rCD4 and incubating them with serial dilutions of the mouse sera starting at 1:100 dilution. Phosphatase-labeled host anti-mouse IgG (obtained from Sigma Chemicals) was used to reveal bound antibodies. Readings were performed in a Titertek automated photometer.

Inhibition of gp120 binding to solid phase CD4. After coating the plates with 3-10 micrograms/ml rCD4, 50 micrograms of gp120 (5 micrograms/ml) were added and incubated for 1 hour, +/- serial two-fold dilution of test antiserum or antibody starting at 100 micrograms/ml concentration. After washing, phosphatase-labeled monoclonal anti-gp120 was applied and incubated 1 hr. The phosphatase activity was then measured by the rate of PNPP hydrolysis. The inhibition caused by the test antibody was expressed in percent decrease from the control gp120 binding.

Effect of mAbs on CD4 binding to solid phase gp120. Plates were coated with 3 micrograms/ml gp120. CD4-phosphatase at 10

micrograms/ml and 3 micrograms/ml were incubated separately for one hour with PTH or with the test mAbs at concentrations 0.3, 1, 3 and 10 micrograms/ml. The mixtures were then added to the coated wells and incubated 1 hr. After washing, the amount of bound CD4 was revealed by PNPP.

Effect of gp120 on CD4 binding to solid-phase captured mAbs.

In separate 96-well plates rgp120 and phosphatase-labeled rCD4 were serially titrated by 10-fold dilution from 80 and 40 micrograms/ml respectively, in different directions on each plate. Equal volumes of each were then combined and incubated 2 hr at room temperatures (RT). Simultaneously with the above incubation, the test monoclonals were added to a goat anti-mouse IgG coated plate for 2 hrs at RT, then washed of excess sample. Fifty microliters of the titrated complex was transferred to the captured mAb-GAM plate and incubated it RT for 2 hrs, then washed. PNPP substrate was added and color developed.

Effect of gp120 on mAbs binding to solid-phase CD4. P l a t e s

were coated with 3/micrograms/ml rCD4; 25 microliters of 0.1-1.0 micrograms/ml rgp120 and 25 microliters of scalar concentrations (0.3-10.0 micrograms/ml) of test mAb were added together to the wells and incubated for 2 hr. After washing, the bound mAb was revealed using goat anti-mouse Ig phosphatase-labeled antibody.

Cross-inhibition of CD4 binding. Competition mapping of mAb specificities was performed by coating plates with rCD4 and layering alkaline phosphatase-labeled mAbs in the absence and presence of graded concentrations of unlabeled test mAbs whose specific binding sites are known from the literature. OKT4A (binding in V1) and OKT4 (binding in V4) were obtained from Ortho (Diagnostics Systems, Raritan, NJ), anti-Leu3a (binding in V1), L83 (V1-V2), L88 (V1-V2), L120 (V4) were a gift from Dr. David Buck, Becton-Dickinson Laboratories, Mountain View, CA.

Inhibition of syncytia formation. The cell fusion among CD4⁺ cell lines acutely infected by the virus requires gp120-CD4 specific binding. The test was performed according to Matthews

et al. (Proc. Natl. Acad. Sci USA 84:5424, 1987). Briefly, GEM cells chronically infected with either HTLV3-III_B or HTLV3-RF were used for each determination. Sera diluted 1:10 were distributed in 96-well A/2 plates (Costar). Five to 10×10^3 HIV-infected cells were added. 70×10^3 uninfected Molt 4 cells were admixed and the plates were incubated overnight at 37°C, after which the number of giant cells (>5 times the size of the parental cells) were counted at 40 X magnification. The mouse serum control varied from 50 to 85 giant cells per well.

Results

1. Polyclonal responses to injection of complexed sCD4-rgp120 in mice.

Three groups of four mice were injected once, either with rCD4 alone (Fig. 2, panels A,D,G), with sCD4-rgp120 complex (Fig. 2, panels B,E,H), or with rgp120 alone (Fig. 2, panels C,F,I). The weekly bleedings were titrated over a 3-month period for capacity to bind CD4 (panels D-F) and gp120 (panels A-C), and for capacity to inhibit the formation of syncytia (panels G-I). The overall results were strikingly different among the three groups. Figure 2 displays the individual titers for each parameter and for each group. There were often multiple peaks during the response (panel D) and mice of a single group showed different timings for their peaks (panels D and H). When the timing and magnitude of the binding and neutralizing responses were examined, (a) there was no clear correlation between the titers of gp120 binding and syncytia blocking (R versus H); (b) there was inverse relationship between rCD4 and rCD4-rgp120 immunized groups when rCD4 binding titers and syncytia blocking were compared (D vs. G and E vs. H), (c) there was a small CD4 binding response in the group immunized with rgp120, which could be attributed to anti-idiotypes (panel F).

2. Polyclonal syncytia-blocking responses by mice receiving CD4-gp120 complexed are not type-specific.

The antisera from mice injected with CD4-gp120 efficiently block syncytia formation caused by such widely different HIV isolates as HTLV3-III_B and HTLV3-RF. Figure 3 shows

individual titrations of the sera from these mice, revealing a similar rank order of the individual responses against the two isolates (93>94>91>92).

- 5 3. The syncytia-blocking capacity of CD4-gp120 responders is absorbed by CD4 and not by gp120.

10 To determine whether the high capacity of sera from complex-immunized mice to inhibit syncytia formation was due to anti-CD4 or anti-gp120 antibodies (or to a cooperative action of the two) a series of absorption experiments was performed using soluble and solid phase bound antigens. The samples were subsequently monitored for changes in binding or syncytia-blocking titers. The results are set forth in Table 1 below.

15

Table 1

Absorption of pooled sera from mice immunized with CD4-gp120, demonstrating that syncytia blocking is associated with anti-CD4.

Absorbent	CD4 binding		gp120 binding		# syncytia**	
	O.D.*	(%)	O.D.*	(%)	IIIB	RF
none	1.216	(100)	.940	(100)	0	3
gp120	1.180	(97)	.188	(20)	0	5
CD4	.420	(34)	.936	(99)	11	24
no antiserum -	-	-	-	-	52	60

*O.D. 405/60 min (color developed by phosphatase hydrolysis of PNPP).

**Syncytia blocking assay as described above.

The results unequivocally attributed the syncytia-blocking capacity to antibodies that recognize the CD4 moiety of the complex rather than anti-gp120, since absorbed anti-gp120 serum showed unaltered syncytia blocking, while the decrease of CD4 binding was accompanied by a significant decrease of syncytia blocking with both isolates tested (Table 1).

4. The capacity to inhibit CD4-gp120 binding in vitro correlates with the CD4-binding titer and not with the syncytia-blocking titer.

Since the syncytia-blocking capacity observed was mediated by anti-CD4, the inverse relationship of binding and syncytia-blocking titers in mice receiving CD4 versus CD4-gp120 had to be attributed to a difference in the fine specificity of the anti-CD4 antibodies in the two groups. To further characterize the anti-CD4 antibodies, one test was to compare their relative capacity to inhibit gp120 binding to CD4. The results are set forth in Table 2 below.

Table 2 Difference in fine specificity distribution among polyclonal anti-CD4 antibodies.

5	CD4 binding (microgram Ab/ml)	Blocking gp120 binding (U ₅₀ /ml)*	Fusion blocking U ₅₀ /ml)	
	Pool #9 (immunized CD4-gp120	2.4	80.0	45.0
10	Pool #18 (immunized CD4)	31.0	890.0	7.0
15				

20 *One blocking U₅₀ is the amount of antibody that reduces to 50% the amount of gp120 bound to CD4-coated wells or, respectively, the number of syncytia in the fusion test. U₅₀/ml was calculated by multiplying 1 U₅₀ unit by the dilution factor for each test.

25 The results (Table 2) showed high inhibition titers in all mice immunized with CD4 alone and low titers in those immunized with the CD4-gp120 complex, indicating that the blocking of syncytia by the latter appeared to be mediated by a mechanism other than prevention of binding of HIV to its receptor on the cell surface.

30 5. Study of anti sCD4-rp120 responses using monoclonal antibodies.

35 In order to dissect the polyclonal response of mice immunized with the CD4-gp120 complex, hybridomas were produced from one of these animals, and the resulting antibodies were characterized for capacity to bind CD4, to bind gp120 and to block syncytia formation. The results are set forth in Table 3 below.

Table 3 List of hybridomas obtained from fusion 91, hierarchically ordered according to their supernatant's capacity to bind CD4, and tested for binding gp120 and blocking syncytia formation.

		<u>Clone Designation</u>	<u>Binding CD4 (O.D.)*</u>	<u>Binding sp120 (O.D.)*</u>	<u># Syncytia at 1/2 dilution**</u>	<u>Syncytia Blocking (%)</u>
10	1	135	1.363	0		
		144	1.208	0		
		148	1.149	.007		
		75	1.059	0		
15		215	1.049	0		
		145	.982	0		
		84	.932	0		
		142	.930	.012		
		140	.937	0		
20	10	210	.724	.015		
		185	.672	0		
		143	.599	.009		
		201	.482	0		
		J	.437	.006		
25		203	.398	.006		
		15	.385	.010		
		156	.372	.028		
		58	.313	.011		
	20	224	.308	0		
30		146	.255	0		
		55	.182	0	9	80
		165	.157	0		
		36	.153	.041	0	100
		172	.145	.006		
35		94	.136	0	0	100
		223	.125	0		
		59	.121	0		
		V	.109	0		
		R	.106	.012		
40	30	48	.106	0		
		35	0	.361		
		40	0	.358		
		M	(.023)	.176		
		68	0	.116		
45		116	(.012)	0	16	65
		95	0	0	18	60
		32	0	0	18	60
					[control 46]	
50	40	.	.	.		
		.	.	.		
		.	.	.		
	170	0	0	0		

*O.D. 405/120 min when the supernatant was tested in ELISA on plates coated with CD4 (respectively, gp120). See methods

above.

** Lower than the control are shown.

Table 3 shows an early test of 170 wells with hybridoma clones, ordered according to their CD4 binding capacity. Thirty (i.e., the majority) of the positive clones produced anti-CD; only four produced anti-gp120, and the remainder were negative for both. Three of the anti-CD4 clones (and none of the anti-gp120) exhibited capacity to block syncytia. All borderline positives (i.e. those showing less than 0.100 in the gp120 binding) and those with partial blocking of syncytia (clones 116, 95 and 32) became negative after subcloning. Table 4 below shows a further characterization of the hybridomas when the inhibition of gp120-CD4 binding test was performed.

Table 4

Classification of mAbs from mice immunized with CD4-gp120 complex.

mAbs:	<u>Binding gp120</u>	<u>Binding CD4</u>	<u>Inhibiting CD4-gp120 Binding*</u>	<u>Group-Specific Syncytia Blocking</u>
48, 35, 40 68	+	-	-	-
135, 144, 148 75, 215, 145 210, 185, 143	-	+	-	-
94, 36	-	+	+	+
55	-	+	-	+

*Cut-off for a positive response was 25% blocking at greater than or equal to a 1:2 dilution.

The anti-CD4 mAbs can be divided into three categories: a) those that do not inhibit gp120 binding and do not block syncytia; b) those that do not inhibit gp120 binding and block syncytia; and c) those that inhibit gp120 binding and block syncytia. MAbs 55 and 94 were further studied, as representatives of the latter two categories, respectively. Both were of IgG₁ isotype.

6. Preliminary mapping experiments. The binding site of both mAbs 55 and 94 was localized within the first two domains (V1-V2) of CD4 by binding experiments using the CD4 IgG immunoadhesin (Genentech) which contains the two external domains of CD4 spliced to an immunoglobulin constant region (data not shown). A cross-inhibition experiment using labeled mAbs 94 and 55, was performed. The binding of these mAbs to solid phase CD4 was tested in the presence of a series of anti-CD4 monoclonal antibodies whose epitopes and binding characteristics are shown or partially known from the literature. The results are set forth in Table 5 below.

Table 5

Cross-inhibition of CD4 binding by mAbs 55 and 94.

		<u>Inhibitor mAbs</u>									
		<u>OKT4A</u>	<u>LEU-3A</u>	<u>L83</u>	<u>L88</u>	<u>L92</u>	<u>L120</u>	<u>OKT4</u>	<u>F91-55</u>	<u>F91-94</u>	<u>F91-36</u>
mAb 55	-	-	+/-	-	-	-	-	-	+	-	+
mAb 94	-	-	-	-	-	-	-	-	-	+	-

*Cut-off point for positivity was 25% or larger decrease in binding when the inhibitor was 6 times more concentrated than the test mAb.

The two mAbs were not inhibited by any of the tested antibodies with the exception of L83, which produced a partial but consistent competition (Table 5). These results do not provide a precise mapping of the binding sites of the two mAbs. OKT4A and anti-Leu3a bind in the first and second domains of CD4 and the test showed that neither binding site overlaps with mAbs 55 and 94. L83 recognizes a conformational determinant which is affected by mutations both in region 8-40 (V1) and region 119-188 (V2). These data taken together indicated that the fine specificity of mAbs 55 and 94 are different from most studied antibodies and different from each other.

7. Interference of gp120 with CD4 binding by mAbs. Preliminary experiments had shown that mAb 94 blocked gp120 binding to CD4, while mAb 55 did not. In order to be able to detect both

inhibition and possible cooperativity between antibody and gp120, a series of three ELISA tests were performed by which the ternary interaction of CD4, antibody and gp120 was examined by keeping in turn one of the reactants in solid phase, and varying the concentrations of the other two. The results are shown in Figures 4, 5 and 6, which also include curves obtained with reference antibodies OKT4A (which competitively interferes with CD4-gp120 binding) and OKT4A (which does not). In Figure 4 the binding of labeled CD4 to gp120 was slightly enhanced or non-significantly changed in the presence of increasing concentrations of 55, while it was progressively inhibited by 94. In Figure 5 the binding labeled CD4 to solid phase-captured antibody was increased 40% by gp120 in the case of 55 at the highest concentration of CD4, but was unaffected or slightly decreased at lower concentrations of CD4. In the case of 94 there was a progressive decrease of CD4 binding in the presence of increased gp120 concentrations, more evident when CD4 was limiting. In Figure 6, the binding of labeled 55 to solid phase CD4 was moderately enhanced in the presence of 0.1 or 1.0 micrograms/ml gp120 while the binding of 94 was depressed in these conditions.

The conclusions of this series of experiments are that a) mAb 94 behaves always as an inhibitor/competitor of the CD4-gp120 binding, and b) mAb 55 in certain conditions does not interfere with the binding in a way similar to OKT4, while in other conditions it showed a degree of cooperativity with CD4-gp120 binding.

Example 5

The effect of immunizing mice with CD4 immunoadhesin (12.5 micrograms), rgp120 (16.5 micrograms) and the combination of CD4 and rgp120 (12.5 micrograms and 16.5 micrograms, respectively) was studied. Three groups of four mice were immunized as described in Example 4 above, and their antibody response to the immunogens was examined 30 days post-immunization using the techniques described in Example 4 above. The results are set forth in Table 6 below.

I M M U N I Z A T I O N

	16.5 microgram gp120			12.5 microgram Immunoadhesin			16.5 microgram gp120 12.5 microgram Immunoadhesin			
5	51	1	3.6	52	1	0	53	1	1.7	gp120 Binding
		2	3.0		2	0		2	3.3	
		3	3.6		3	0		3	3.7	
		4	3.2		4	0		4	3.4	
10	55	1	4.3	57	1	0	56	1	4.8	
		2	2.8		2	0		2	3.6	
		3	3.8		3	0		3	3.0	
		4	4.3		4	0		4	3.3	
15	<hr/>									
	51	1	0	52	1	2.0	53	1	2.0	CD4 Binding
		2	0		2	3.9		2	2.0	
		3	0		3	1.7		3	2.4	
20		4	0		4	2.0		4	2.3	
	55	1	0	57	1	3.3	56	1	3.8	
		2	0		2	4.0		2	3.7	
		3	0		3	4.0		3	3.9	
25		4	0		4	3.9		4	3.6	
30	<hr/>									
	51	1	0	52	1	0	53	1	1.0	Syncytia Blocking
		2	0		2	0		2	1.9	
		3	0		3	0		3	2.2	
		4	0		4	0		4	1.6	
	55	1	0	57	1	0	56	1	1.6	
35		2	0		2	0		2	1.3	
		3	0		3	0		3	0	
		4	0		4	0		4	1.0	

As can be seen in the data set forth in Table 6 above, mice receiving the complex Immunoadhesin-rgp120 responded to gp120 with antibody titers similar to those receiving only rgp120 while these mice responded to CD4 with antibody titers similar to those receiving Immunoadhesin alone. However, mice which were immunized with Immunoadhesin-rgp120 were the only group of mice which showed significant-titers of syncytia-blocking antibodies.

All cited literature and patents or patent applications are incorporated by reference in their entirety.

Appendix of Materials Sources

5	CD4, CD4-Immunoaderesin	Genentech Inc., South San Francisco, CA
	Freund's Adjuvant	Difco Laboratories, Surrey, England
10	goat antimouse immunoglobulin	Sigma Chemical Co., St. Louis, Missouri
	gp120	Genentech Inc., South San Francisco, CA
	microtiter plates	Linbro available from Fisher Scientific, Springfield, N.J.
15	96A half-well plates	Costar available from Fisher Scientific Springfield, N.J.
20	PNPP	Sigma Chemical Co., St. Louis, Missouri
	Tween	Sigma Chemical Co., St. Louis, Missouri
25	Titertrek Multiskan Apparatus	Flow Laboratories, McLean, Virginia
	RPMI 1640	GIBCO, Grand Island, N.Y.

WHAT IS CLAIMED IS:

1 1. A method for inhibiting cell fusion between human
2 T-lymphocytes infected by human immunodeficiency virus and
3 uninfected healthy human T-lymphocytes, the method comprising:

4 exposing said healthy lymphocytes prior to their
5 binding to said infected lymphocytes to the presence of an
6 inhibitory effective amount of antibodies that have been raised
7 against a complex, said complex comprising (a) HIV protein
8 gp120 and (b) a member selected from the group consisting of
9 (i) CD4-Immunoaderhin, (ii) cell-free CD4 and (iii) an-
10 tiidiotypic antibody to CD4 bearing a CD4 internal image.

1 2. A method for inhibiting invasion of human T-
2 lymphocytes by human immunodeficiency virus (HIV) the method
3 comprising:

4 prior to or after binding of said virus and said
5 lymphocyte, exposing said virus to the presence of an in-
6 hibitory effective amount of antibodies that have been raised
7 against a complex, said complex comprising (a) HIV protein
8 gp120 and (b) a member selected from the group consisting of
9 (i) CD4-Immunoaderhin, (ii) cell-free CD4 and (iii) an-
10 tiidiotypic antibody to CD4 bearing a CD4 internal image.

1 3. A method for raising antibodies that inhibit cell
2 fusion between uninfected human lymphocytes and at least one of
3 (a) human lymphocytes infected with human immunodeficiency
4 virus (HIV) and (b) HIV virus particles, the method comprising:

5 immunizing a mammal with both HIV protein gp120
6 and a member selected from the group consisting of (i) CD4-
7 Immunoaderhin, (ii) cell-free CD4 and (iii) antiidiotypic
8 antibody to CD4 bearing a CD4 internal image.

9 waiting for said mammals to mount an immune
10 response; and

11 collecting said antibodies.

1 4. The method of claim 3 comprising immunizing said

2 mammal with a mixture of said gp120 and said member.

1 5. The method of claim 3 comprising simultaneously
2 immunizing said mammal with said gp120 and said member.

1 6. The method of claim 3 comprising successively
2 immunizing said mammal with said gp120 and said member.

1 7. An immunogenic composition comprising as an active
2 ingredient an immunogenically effective amount of a complex of
3 gp120 and a member selected from the group consisting of (i)
4 CD4-Immunoaderhin, (ii) cell-free CD4 and (iii) antiidiotypic
5 antibody to CD4 bearing a CD4 internal image.

1 8. The composition of claim 7 further comprising a
2 physiologically acceptable medium.

1 9. The composition of claim 7 further comprising an
2 immunization adjuvant.

1 10. The composition of claim 7 further comprising an
2 immunogenicity-enhancing carrier.

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FIG. 1C

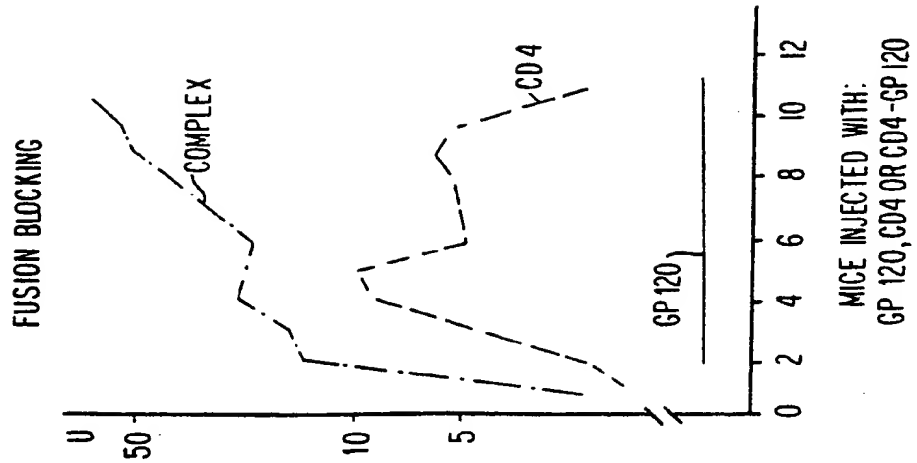


FIG. 1B

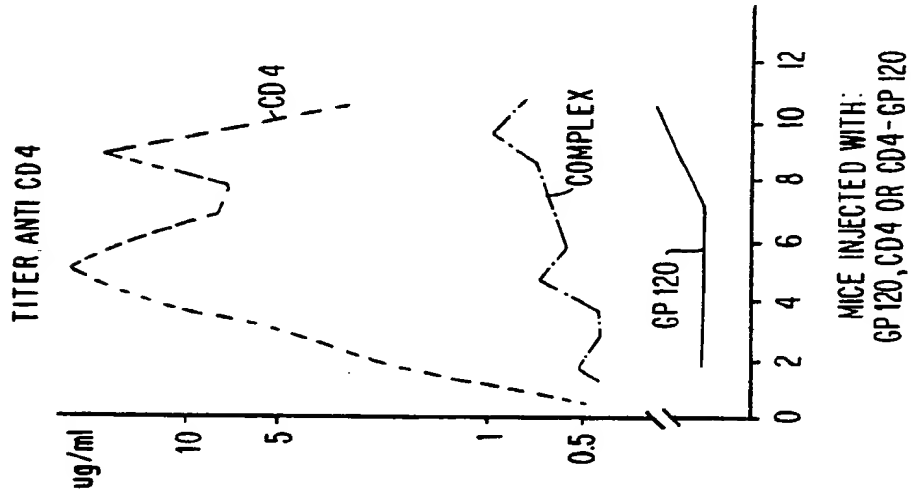
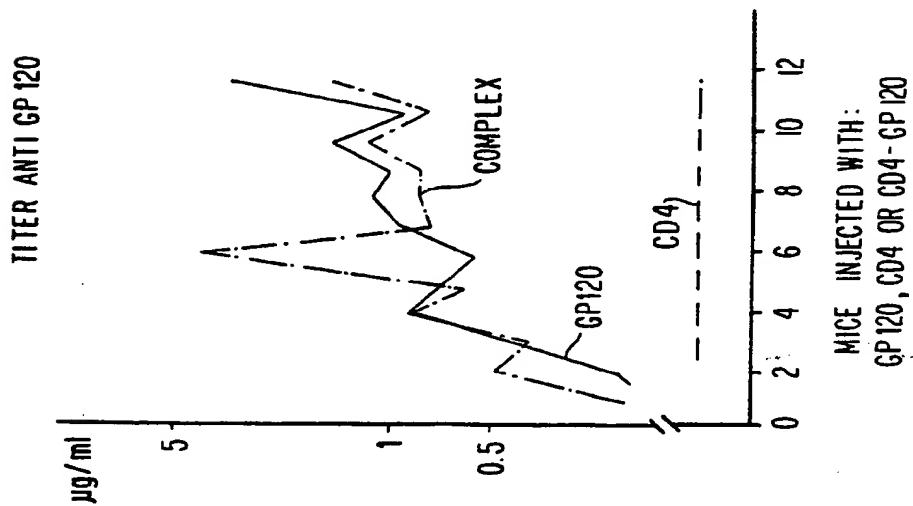


FIG. 1A



SUBSTITUTE SHEET

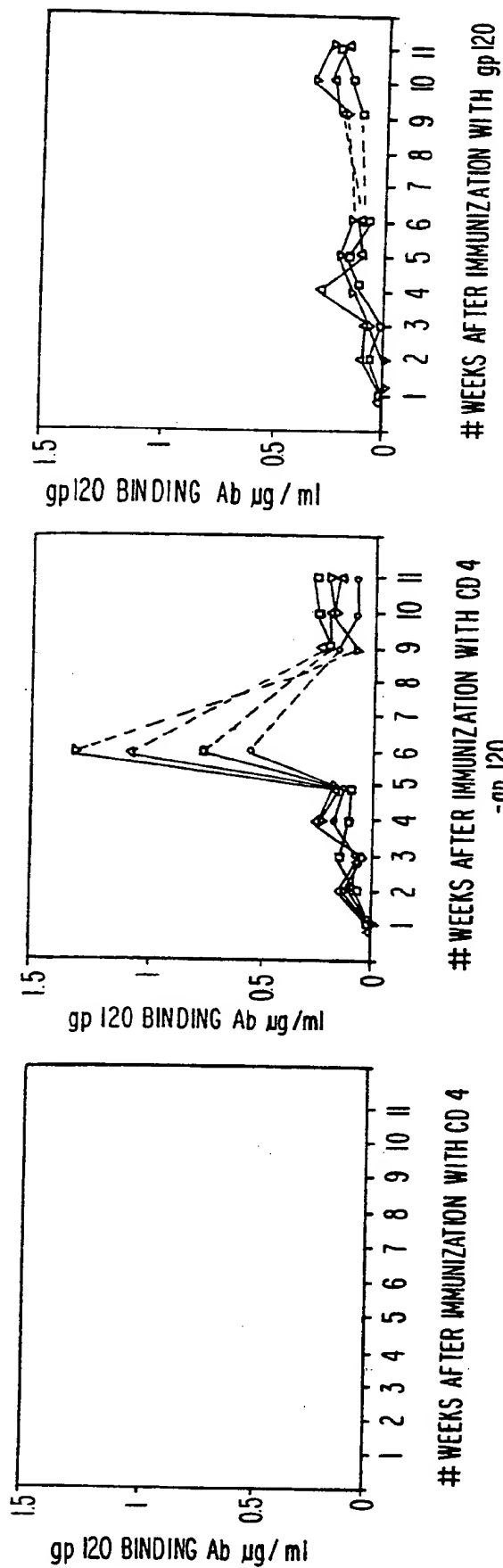


FIG. 2C

FIG. 2B

FIG. 2A

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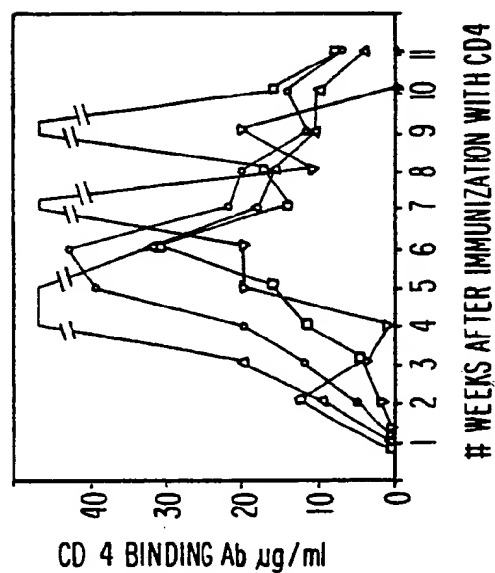
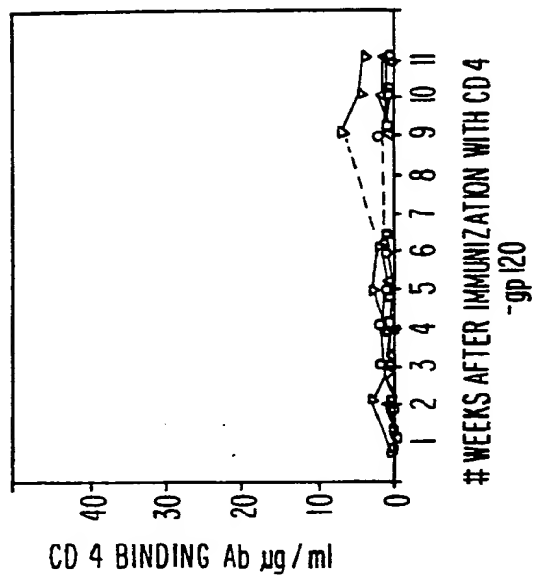
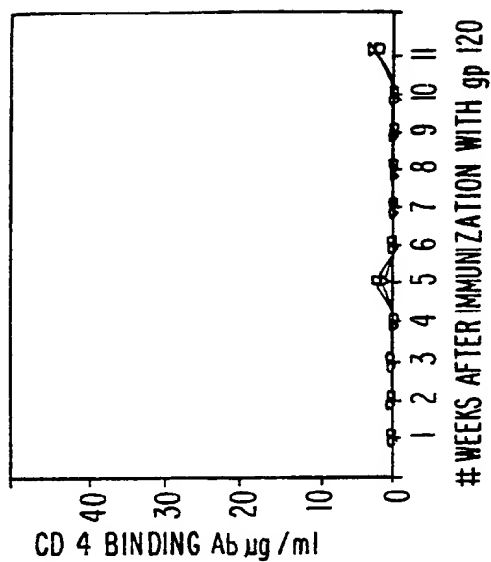


FIG. 2F

FIG. 2E

FIG. 2D

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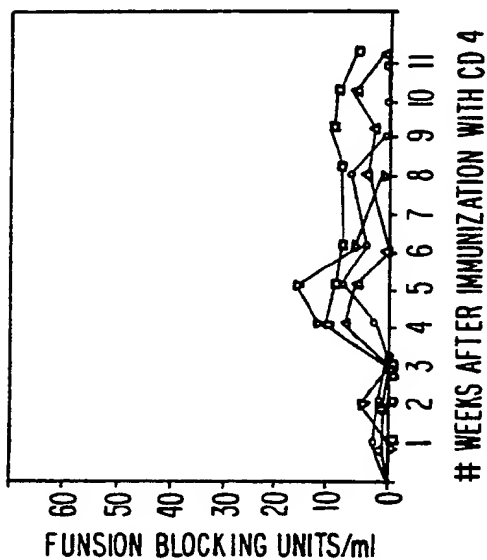
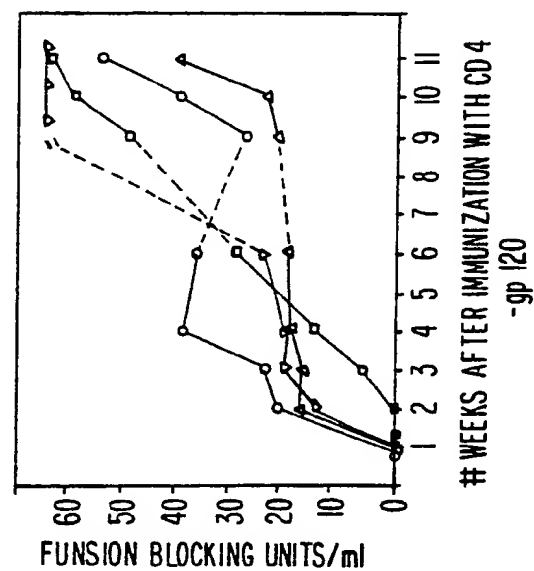
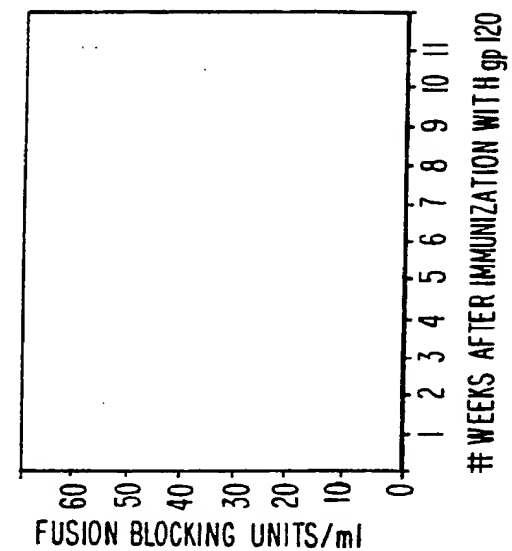


FIG. 2I

FIG. 2H

FIG. 2G

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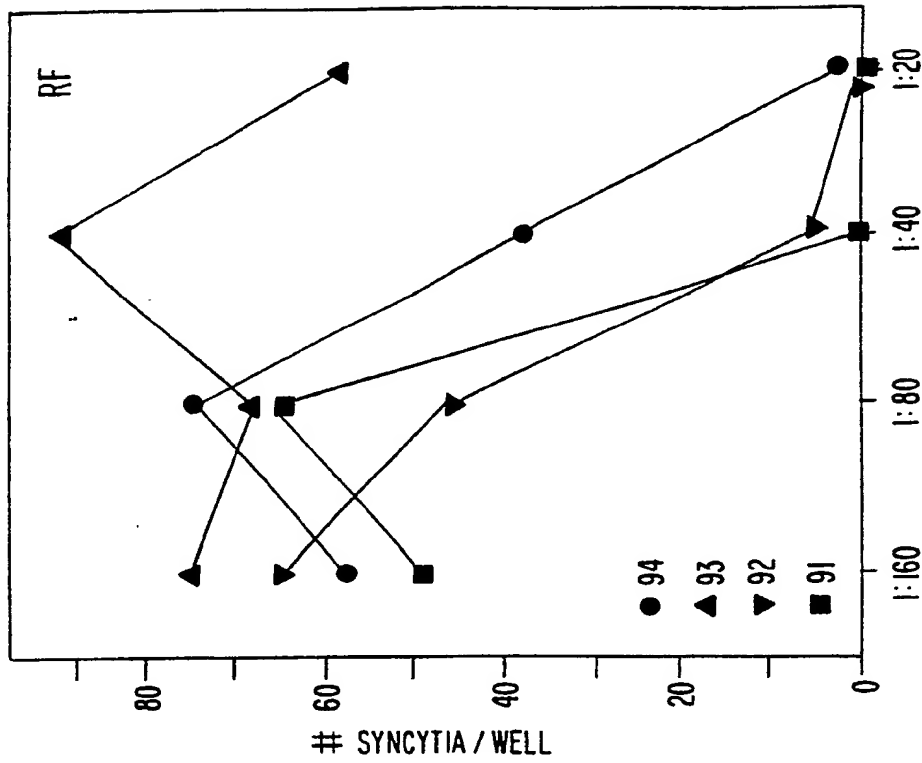


FIG. 3B

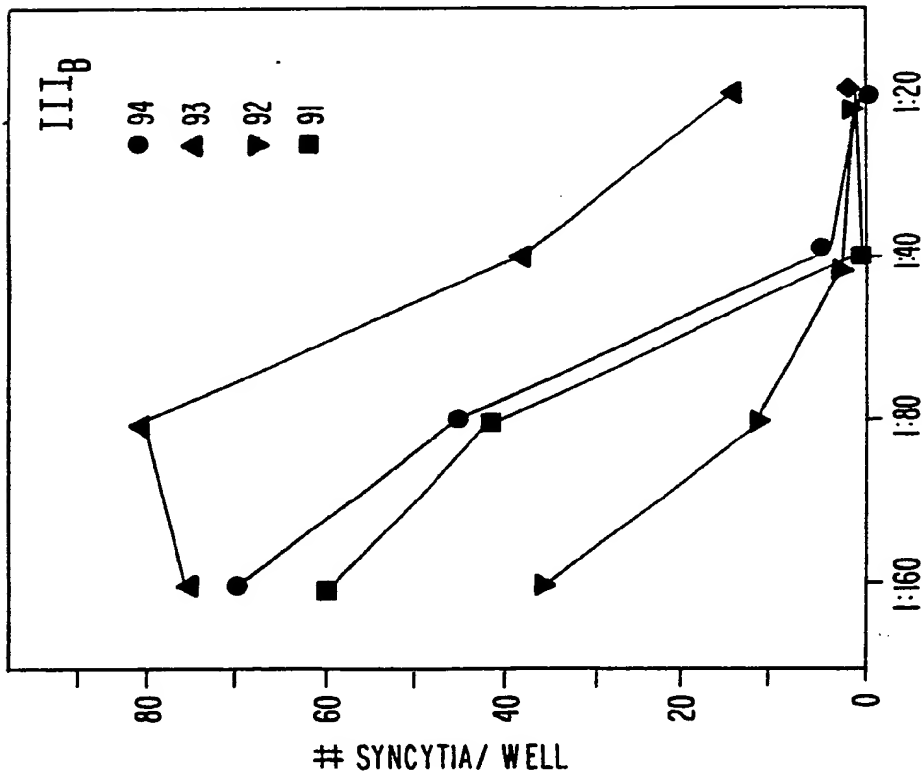
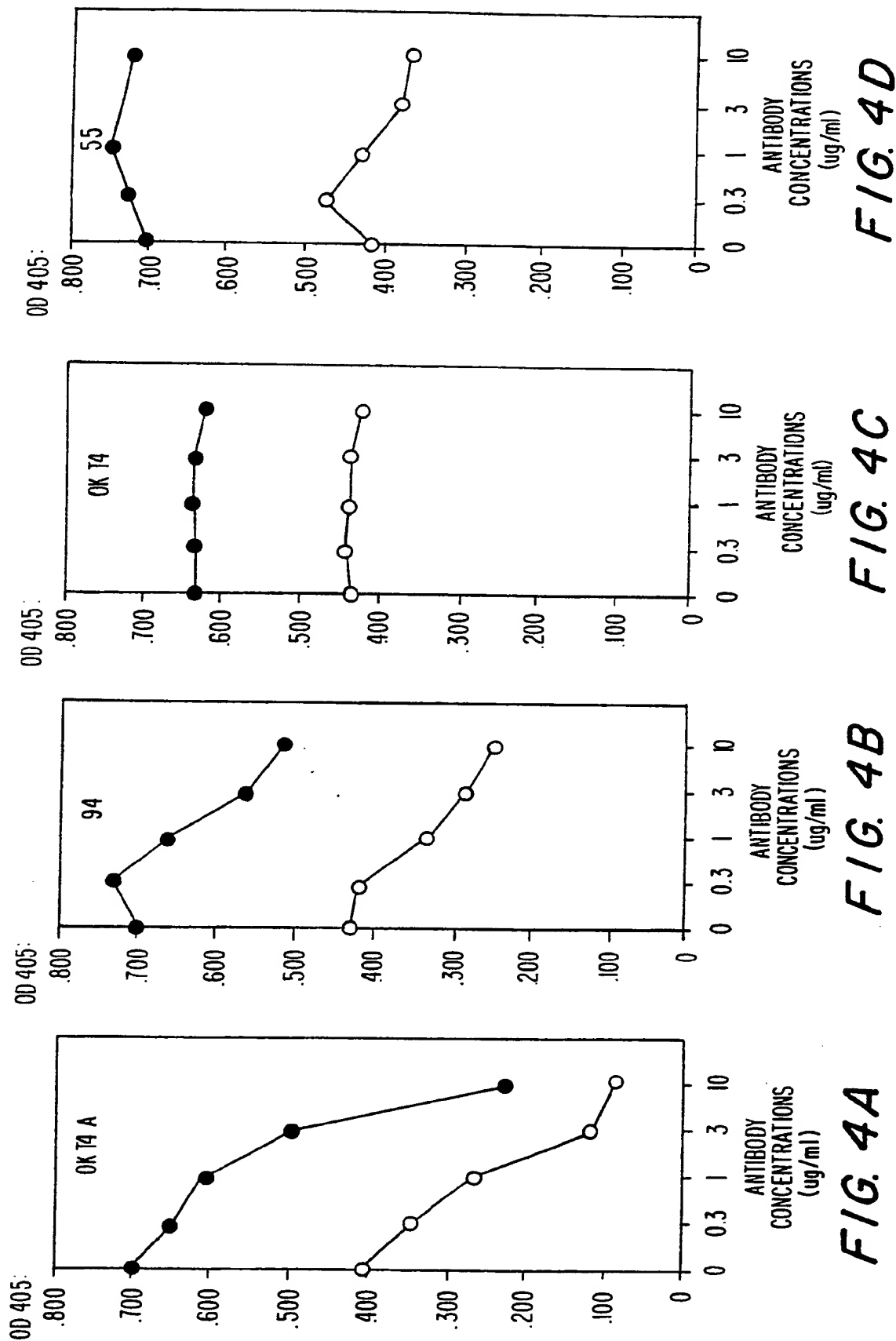


FIG. 3A

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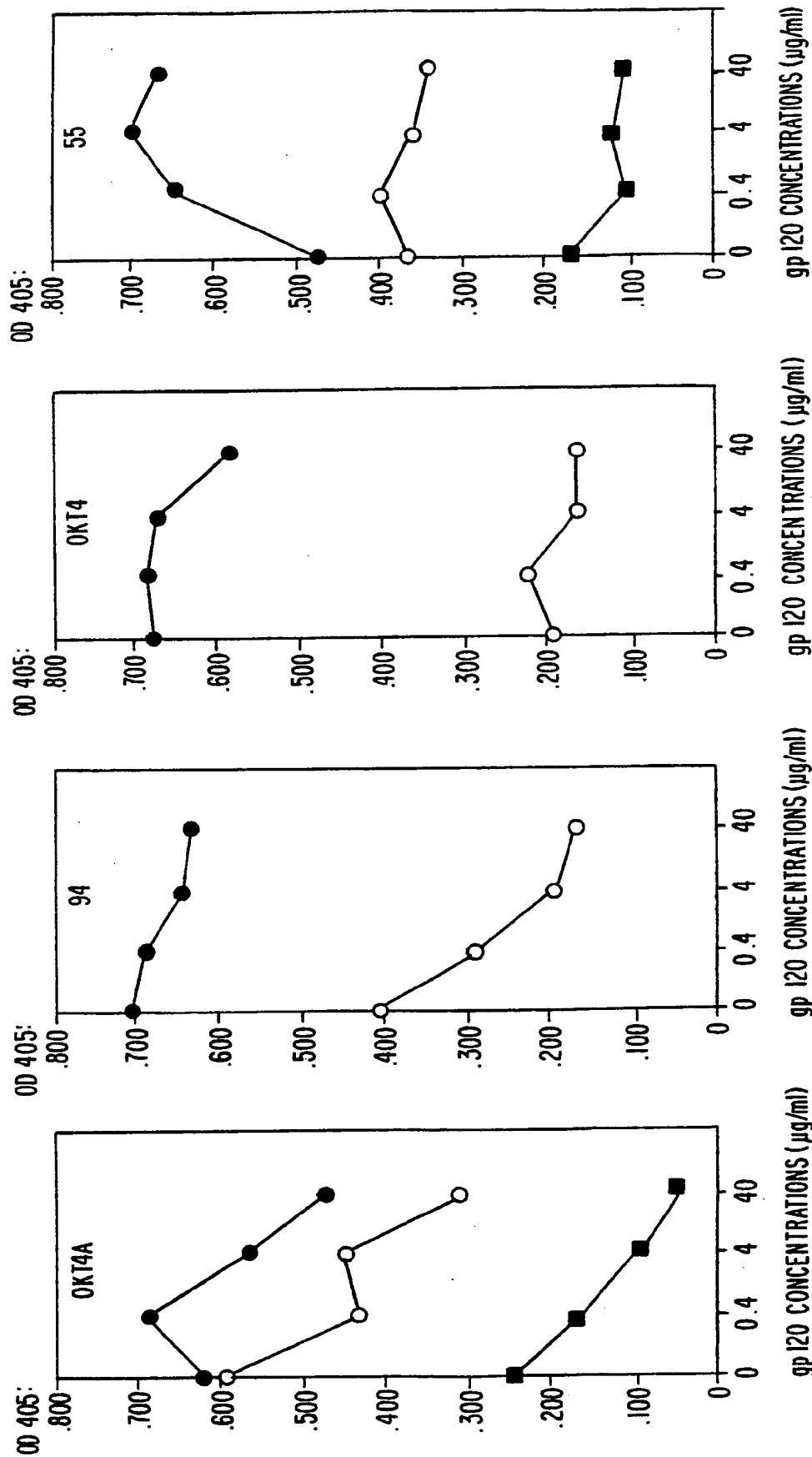


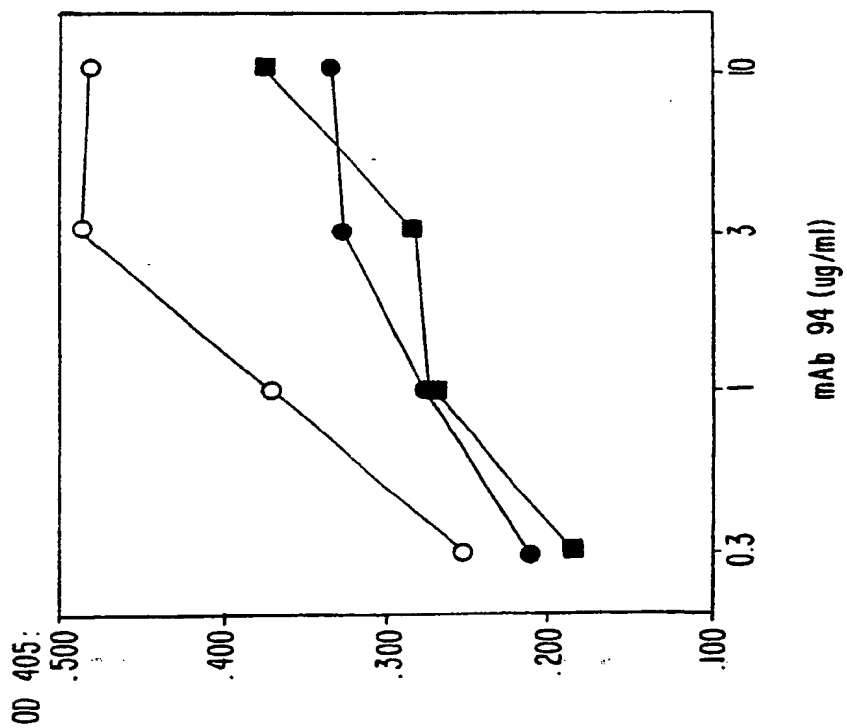
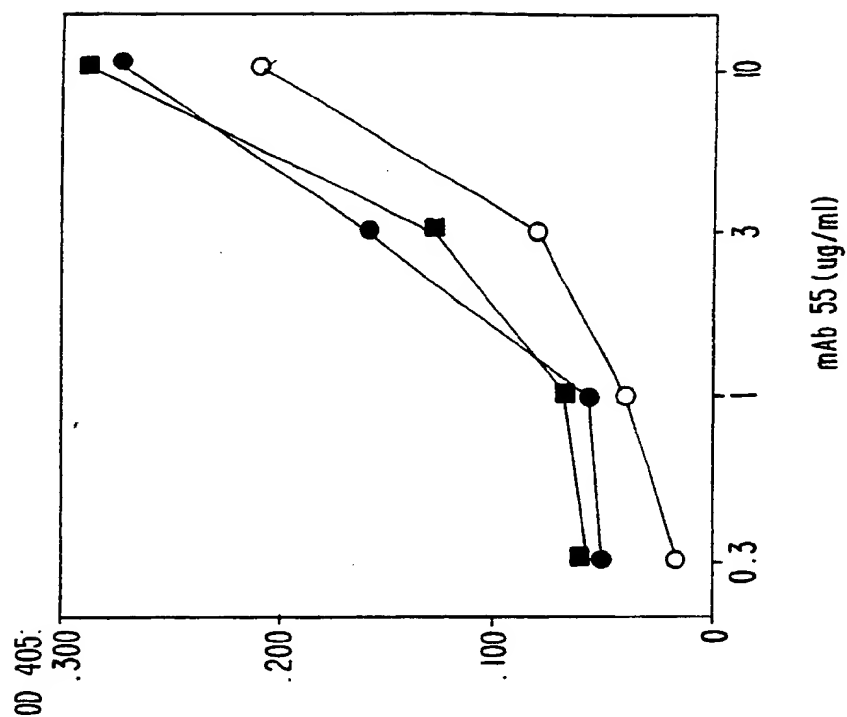
FIG. 5D

FIG. 5C

FIG. 5B

FIG. 5A

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06079

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 39/12; C07K 15/00 USC1.: 424/89; 530/350		
II. FIELDS SEARCHED Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	424/89; 530/350	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Databases: Dialog (Files 5, 154, 155, 157, 399, 357), USPTO Automated Patent System (File USP?AT, 1971-1991).		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Nature, vol. 337, issued 09 February 1989, Capon, et al., "Designing CD4 Immunoadhesins for AIDS Therapy", pages 525-531, ^{see} _A entire document.	1-10
Y	Proceedings National Academy of Sciences, vol. 85, issued April 1988. Berger, et al., "A Soluble recombinant polypeptide comprising the Amino-terminal Half of the Extracellular Region of the CD4 Site for Human Immunodeficiency Virus", pages 2357-2361, see abstract.	1-10
Y	Nature, vol. 331, issued 07 January 1988. Hussey et al., "A Soluble CD4 Protein Selectively Inhibits HIV Replication and Syncytium Formation", pages 73-81, see entire document.	1-10
<p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 15 October 1991 International Searching Authority ISA/US		Date of Mailing of this International Search Report 16 DEC 1991 Signature of Authorized Officer <i>Lynette F. Smith</i> Lynette F. Smith

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Virology, vol. 63, no. 9, issued September 1989, Sun et al., "Generation and Characterization of Monoclonal Antibodies to the Putative CD4-Binding Domain of Human Immunodeficiency Virus Type 1 Sp120", pages 3579-3585, see abstract....	1-10